OUTGROWTH OF *CLOSTRIDIUM PERFRINGENS* SPORES IN COOK-IN-BAG BEEF PRODUCTS[1]

VIJAY K. JUNEJA[2] and WENDY M. MAJKA

Microbial Food Safety Research Unit
USDA/ARS Eastern Regional Research Center
600 E. Mermaid Lane, Philadelphia, PA 19118

ABSTRACT

Clostridium perfringens *growth from a spore inoculum was investigated in vacuum-packaged, cook-in-bag ground beef that included 0.3% (w/w) sodium pyrophosphate, pH 5.5 or 7.0, and salt (sodium chloride) at 0 or 3% (w/w). The packages were processed to an internal temperature of 71.1C, ice chilled and stored at various temperatures. The total* C. perfringens *population was determined by plating diluted samples on tryptose-sulfite-cycloserine agar followed by anaerobic incubation for 48 h at 37C. At 28C, a combination of 3% salt and pH 5.5 was effective in delaying growth for 24 h. At 15C, growth occurred within 6 days in samples with pH 7.0, but was delayed until day 8 in the presence of 3% salt at pH 7.0. Vegetative cells were not observed even after 21 days of storage at 15C in the presence 3% salt at pH 5.5.* C. perfringens *growth was not observed at 4C regardless of pH or salt levels. The D-values ranged from 23.33 min (3% salt, pH 7.0) to 13.99 min (3% salt, pH 5.5). Cyclic and static temperature abuse of refrigerated products for 12–15 h did not permit* C. perfringens *growth. However, temperature abuse of cook-in-bag beef products for periods longer than 15 h in the absence of salt led to growth of* C. perfringens *from a spore inoculum.*

[1]Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.
[2]Author to whom correspondence should be addressed. Telephone: (215) 233-6500.

INTRODUCTION

The presence of *C. perfringens* spores in foods is a potential health hazard. Vegetative cells are killed at 65C, but the heat processes involved in cooking foods may heat-shock spores with resultant germination, outgrowth and vegetative cell growth to unsafe levels under favorable conditions (Genigeorgis 1975; Hauschild 1973; Labbe 1989; Juneja *et al.* 1994). Such favorable conditions include storage for periods greater than 12 h at abusive temperatures of $\geqslant$ 28C (Juneja *et al.* 1994). The incidence of *C. perfringens* food poisoning has been high in recent years and cooked beef has been frequently associated with disease outbreaks (Bryan and McKinley 1979; Bryan 1969, 1988). In the 15 year period, 1973–1987, the organism accounted for 26.8% of the gastroenteritis outbreaks when cooked beef was implicated as the vehicle of transmission (Bean and Griffin 1990). Food poisoning results after the ingestion of foods containing large populations of viable vegetative cells of *C. perfringens*, which produce an enterotoxin during sporulation in the intestine (Labbe 1989). The enterotoxin is responsible for typical symptoms of illness (Stark and Duncan 1971). Although the illness is typically mild (abdominal pain and diarrhea lasting 12–24 h), fatalities may occasionally occur in debilitated patients, especially the elderly.

Vacuum-packaged, cook-in-bag beef products (*sous vide* food) meet consumers' demand for fresh or nearly fresh products requiring minimal preparation time. There has been an increased interest in heat processing of food under vacuum in air-impermeable bags (Light *et al.* 1988; Light and Walker 1990; Smith *et al.* 1990). But, the application of such technologies has been limited in the retail environment because of public health safety concerns (Shamsuzzaman *et al.* 1992). On the other hand, in USDA processing plants, millions of pounds of vacuum packaged, pasteurized roast beef, turkey, and chicken are produced annually following 9CFR 318.17 without incident. Because the applied heat treatment does not produce commercial sterility, the products have to be transported, distributed, stored and handled under refrigeration to inhibit heat resistant spoilage and pathogenic microorganisms, and ensure product safety (Light *et al.* 1988). There is a potential for *C. perfringens* spore germination and vegetative cell growth, and concern is justified in view of the reports that some *C. perfringens* strains can grow at temperatures as low as 6C (Johnson 1990). Surveys of retail food stores and consumer refrigeration units have revealed that holding temperatures of $>$ 10C are common (Daniels 1991; Bryan *et al.* 1978; Wyatt and Guy 1980; Hutton *et al.* 1991; Anon. 1989; van Grade and Woodburn 1987). Moreover, temperature abuse during postprocess handling and storage is the most common cause of food poisoning associated with *C. perfringens* (Bean and Griffin 1990). Since temperature abuse is a common occurrence at both the retail and consumer levels, the Refrigerated Food and Microbiological Criteria Committee of the Na-

tional Food Processors Association (1988) has recommended that additional safety barriers be incorporated in refrigerated foods.

To our knowledge, the effects of the *sous vide* process on *C. perfringens* spores and the fate of spores during storage of foods treated by the *sous vide* have not been reported. Therefore, the present work was undertaken to investigate (1) the effect of cyclic and static temperature abuse of refrigerated beef samples on *C. perfringens* spore germination and multiplication of vegetative cells in vacuum-packaged, cook-in-bag ground beef and (2) to provide information on the thermal inactivation of *C. perfringens* spores in beef slurry.

MATERIALS AND METHODS

Test Organisms and Spore Production

Clostridium perfringens strains NCTC 8238, NCTC 8239 and ATCC 10288 from the Microbial Food Safety culture collection were maintained as sporulated stock cultures in cooked meat medium (Difco Laboratories, Detroit, MI). An active culture was prepared in freshly prepared fluid thioglycollate medium, and sporulation was carried out in Duncan and Strong sporulation medium as previously described (Juneja *et al.* 1993). After the spore crop of each strain had been washed twice and resuspended in sterile distilled water, the spore suspensions were stored at 4C. A spore cocktail containing all three strains of *C. perfringens* was prepared immediately prior to experiments by mixing equivalent numbers of spores from each suspension. This composite of spore strains was heat-shocked for 20 min at 75C prior to use.

Source, Preparation and Inoculation of Meat

Ground beef was obtained from a local retail market and frozen (−5C) until use (approximately 40 days). Sodium pyrophosphate was mixed into all ground beef samples with a Hobart mixer to give a final concentration of 0.3% (w/w). All combinations of salt (0 or 3%, w/w) and pH (5.5 or 7.0) were replicated twice. The pH of the ground beef was determined using a combination electrode (Sensorex, semi-micro, A.H. Thomas, Philadelphia, PA) attached to an Orion model 601A pH meter and was adjusted with lactic acid. Duplicate 25 g ground beef samples were aseptically weighed into filter stomacher bags (SFB-0410; Spiral Biotech., Bethesda, MD) and inoculated with the heat-shocked *C. perfringens* spore cocktail so that the final concentration of spores was approximately 3 $\log_{10}$ cfu/g. Thereafter, the bags were manually mixed to ensure even distribution of the organisms in the meat sample. Negative controls consisted of bags contain-

ing uninoculated beef. The bags were placed in 7″ × 8″ plastic barrier bags (Koch Model 01 46 09, Kansas City, MO). The oxygen transmission rate of the nylon/polyethylene film was 3.5 cc/100 in.^{2} in 24 h measured at 75F and 75% relative humidity. The bags were evacuated to a negative pressure of 1000 millibars and heat sealed using a Multivac Model A300/16 gas packaging machine (W. Germany).

Cooking and Cooling Protocols

Prior to cooking, two representative bags were opened, sterile copper-constantan thermocouples were placed at the center of each of the ground beef samples and the bags were resealed. The ground beef samples were fully submerged in 80C water in a water circulating bath (Exacal, Model Ex-251HT, NESLAB Instruments, Inc., Newington, NH). The internal temperature of the samples was constantly monitored by thermocouples. The readings were measured and recorded by a Keithly-Metrabyte data logger connected to a microcomputer. The thermocouple signal was sampled every second (0.02 min), and the two readings were averaged to determine the sample temperature. The samples were processed to an internal temperature of 71.1C within 10 min and quickly cooled in an ice slurry.

Storage, Temperature Abuse, and Sampling

The inoculated samples were stored at 4, 15, and 28C. Samples stored at 28C were analyzed at 3, 6, 9, 12, 18, 24, 36 and 48 h, those at 15C were analyzed on day 1, 2, 3, 4, 6, 8, 10, 14 and 21 and the samples stored at 4C on day 7, 14, 21, and 28. To determine the effect of static temperature abuse, some samples stored at 4C were moved 7 days before plating on their scheduled sampling day (7, 14, 21, and 28 days) to 15C, and some 4C samples were transferred 15 and 20 h to 28C before sampling. To determine the effect of cyclic temperature abuse, samples stored at 4C were moved 7 days before plating on their scheduled sampling day (7, 14, 21, and 28 days) to 28C, held at this temperature for 12 or 24 h, and then returned to 4C and plated on their scheduled sampling day.

Bacterial Enumeration Procedure

On the scheduled sampling day, samples were removed and enumerated for total *C. perfringens* population by spiral plating (Spiral Systems Model D plating instruments; Cincinnati, OH) on tryptose-sulfite-cycloserine (TSC) agar as described previously (Juneja *et al.* 1994). The total *C. perfringens* population was determined after 48 h of incubation at 37C in a Gas Pak system (Baltimore Biological Laboratory, Cockeysville, MD). In addition, a 25 g portion of both

uninoculated raw beef and cooked beef were used to verify the absence of naturally occurring *C. perfringens*. This involved the use of lactose-gelatin and nitrate-motility medium (Schwab *et al.* 1984).

Determination of Spore Heat Resistance

A known weight of beef was aseptically transferred to a sterile Waring Blendor and mixed with an equal volume of sterile distilled water by blending for 2 min to form a smooth paste. Beef slurry containing 0.3% (w/w) sodium pyrophosphate at 4 treatment combinations (pH 5.5 or 7 and salt, 0 or 3%, w/w) was inoculated with the heat-shocked *C. perfringens* spore cocktail to obtain an initial count of about 7 $\log_{10}$ spores/ml. Inoculated samples were blended to ensure uniform distribution of spores. The mixture was dispensed in 10 ml portions to sterile 17 × 60 mm screw-capped vials. Negative controls included vials containing uninoculated beef slurry. Each vial was sealed with a sterile lid fitted with rubber septa and fully submerged in a temperature controlled water bath (Exacal, Model Ex-251HT) stabilized at 99C. The temperature was continuously monitored by two copper-constantan thermocouples inserted at the center of two uninoculated vials. Once the meat slurry reached the desired temperature, two vials were quickly removed and cooled by swirling in an ice slurry. The average number of *C. perfringens* spores surviving in these samples was the number present at zero time. Thereafter, two vials were taken at fixed heating time intervals, cooled in ice and the surviving spore population was determined by enumerative spiral plating onto dishes containing TSC with lysozyme (10 μg/ml; Sigma, 41,000 U/mg), followed by anaerobic incubation at 28C for 6 days.

Data Processing

Bacterial growth curves were generated from the experimental data using the Gompertz equation (Gibson *et al.* 1987) in conjunction with ABACUS, a nonlinear regression program that employs a Gauss-Newton iteration procedure. This FORTRAN-based program was developed by W.C. Damert (U.S. Department of Agriculture, Eastern Regional Research Center, Philadelphia, PA). The Gompertz parameter values were subsequently used to calculate generation times and lag times as described by Gibson *et al.* 1987. The D-values (time for 10-fold reduction in viable spores) were estimated by computing the linear regression (Ostle and Mensing 1975) of $\log_{10}$ number of survivors versus heating time using Lotus 1-2-3 Software (Lotus Development Corporation, Cambridge, MA) and taking the absolute value of the inverse slope. The heat resistance data were analyzed by analysis of variance (ANOVA) using SAS (SAS 1989) to determine if there were any statistically significant differences among the treatments. A 2

× 2 factorial arrangement in a completely randomized design with two replicates was employed, with salt levels (0 and 3%) and pH (5.5 and 7) as main effects. Since the pH and salt interaction was significant ($p < 0.05$), main effects were not reported. Bonferroni mean separation test was used to determine significant differences ($p < 0.05$) among means (Miller 1981).

RESULTS AND DISCUSSION

During storage at 28C, *C. perfringens* spores germinated and grew from 3 to approximately 5 $\log_{10}$ cfu/g after 18 h in cooked ground beef products (pH 7) regardless of the presence or absence of salt. Similar results were obtained for ground beef at pH 5.5 with no salt (Fig. 1). A combination of 3% salt and pH 5.5 delayed *C. perfringens* growth for 24 h at 28C. Thereafter, germination and multiplication of vegetative cells occurred at a relatively slow rate and the total *C. perfringens* population was < 5 $\log_{10}$ cfu/g even after 48 h of storage at 28C at low pH and in the presence of salt (Fig. 1). At 28C, the generation times ranged from 80.1 min in salt-free beef samples at pH 7.0 to 129.2 min in samples at pH 5.5 with 3% salt (Table 1). The lag times were 11.55 h and 27.53 h, respectively. Juneja *et al.* (1994) reported a longer generation time (118.9 min) and a shorter lag time (7.06 h) of *C. perfringens* vegetative cells inoculated in cooked beef (pH 6.25) before vacuum packaging and storing at 28C. Pivnick *et al.* (1968) cooked chicken containing 4 $\log_{10}$ spores/g for barbecuing to an internal temperature of 85 to 90C and stored at 45C. They reported a lag period of 4 h followed by logarithmic growth which proceeded at the same rate as that observed for vegetative cells in inoculated samples.

By day 6 at 15C, *C. perfringens* spores germinated and grew to > 5 $\log_{10}$ cfu/g in beef samples at pH 7.0 with no salt (Fig. 1). In the presence of 3% salt, growth was relatively slow and took 10 days to increase to > 5 $\log_{10}$ cfu/g. In samples at pH 5.5, growth was considerably delayed at 15C in the absence of salt, but in the presence of 3% salt, vegetative cells were not observed in beef samples even after 21 days of storage (Fig. 1). In contrast to 28C, *C. perfringens* exhibited 5 × longer generation time (415.9 min) and 8 times longer lag time (96.06 h) at 15C in samples at pH 7.0 with no salt (Table 1). Juneja *et al.* (1994) studied the fate of the *C. perfringens* vegetative cells in vacuum-packaged cooked ground beef (pH 6.25) at 15C and reported generation and lag times as 2148 min and 112.56 h, respectively.

During storage at 4C, *C. perfringens* growth from a spore inoculum was not observed in beef samples regardless of pH or salt levels (Fig. 2; data with salt or pH 5.5 not shown). Similar observations were reported by Cooksey *et al.* (1993) who pasteurized beef in a water bath to an internal temperature of 60C for 16 min and stored at 4C.

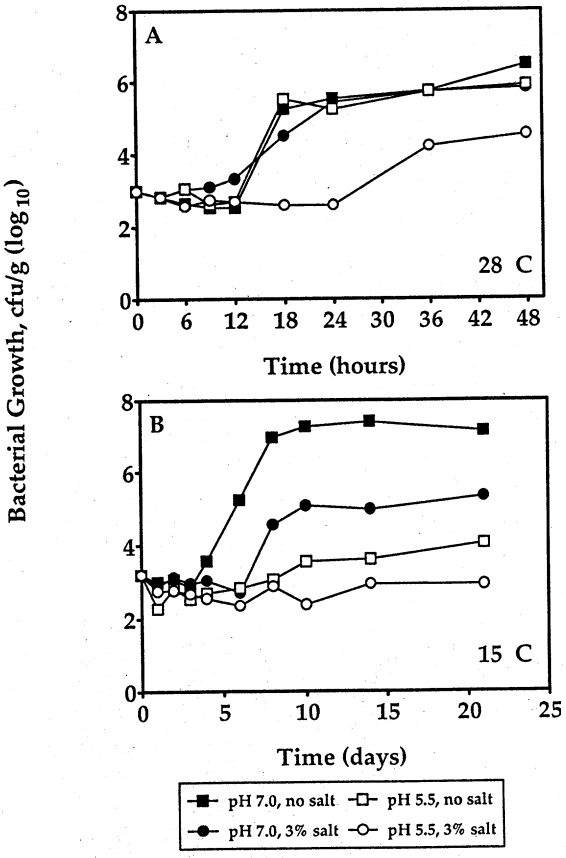

FIG 1. THE EFFECT OF TEMPERATURE ABUSE (STORAGE AT 15 OR 28C) ON GROWTH OF *CLOSTRIDIUM PERFRINGENS* FROM A SPORE INOCULUM IN VACUUM-PACKAGED, COOK-IN-BAG GROUND BEEF THAT INCLUDED 0.3% SODIUM PYROPHOSPHATE AT pH 5.5 OR 7.0, AND SALT LEVELS 0 OR 3%

The National Food Processors Association (NFPA 1988) indicated manufacturers should assume that temperature abuse will occur at some point during the distribution of a refrigerated food product. To determine the effect of static temperature abuse of refrigerated beef products which may occur during transportation, distribution, storage or handling in supermarkets or by consumers, beef samples stored at 4C were moved to 15C, 7 days before plating on their scheduled sampling day (7, 14, 21, and 28 days). *C. perfringens* spores germinated, and grew to > 7 $\log_{10}$ cfu/g only in samples at pH 7 with no salt (Fig. 2). When samples were transferred to 28C, 15 h before plating on their scheduled sampl-

TABLE 1.
MEAN GENERATION TIMES AND LAG TIMES[a] OF *CLOSTRIDIUM PERFRINGENS* IN GROUND BEEF THAT INCLUDED 0.3% SODIUM PYROPHOSPHATE AT pH 5.5 OR 7.0 WITH (3%) OR WITHOUT SALT

Beef product		Generation Times (min)[b]		Lag Times (h)	
pH	Salt (%)	28C	15C	28C	15C
7	0	80.1	415.9	11.55	96.06
7	3	88.8	439.0	16.58	159.06
5.5	0	122.1	4640.7	12.83	200.52
5.5	3	129.2	NG	27.53	NA

[a]Means represent two replications.
[b]Generation times calculated from regression lines for exponential growth using the Gompertz equation.

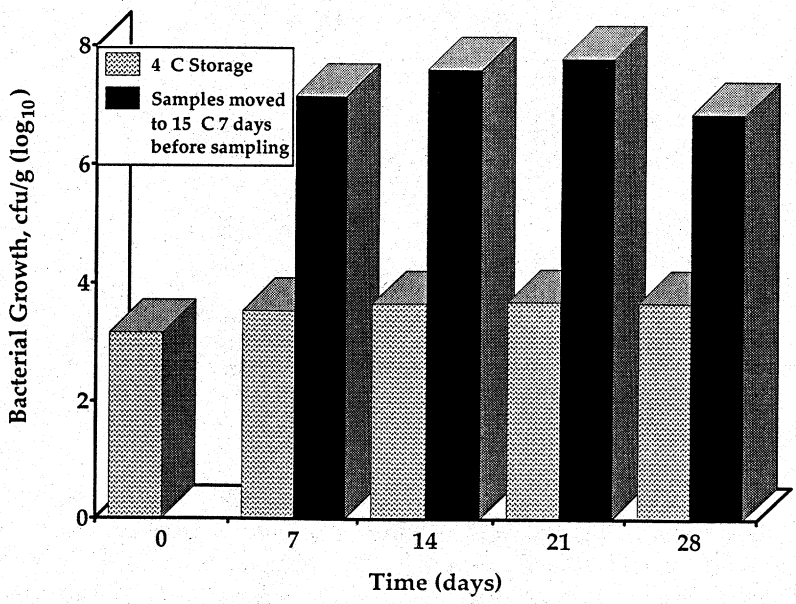

FIG. 2. THE EFFECT OF STATIC TEMPERATURE ABUSE ON GROWTH OF *CLOSTRIDIUM PERFRINGENS* FROM A SPORE INOCULUM IN VACUUM-PACKAGED, COOK-IN-BAG GROUND BEEF (pH 7) THAT INCLUDED ONLY 0.3% SODIUM PYROPHOSPHATE AND STORED AT 4C
The samples were moved to 15C for 7 days before plating on their scheduled sampling day (7, 14, 21, and 28 days).

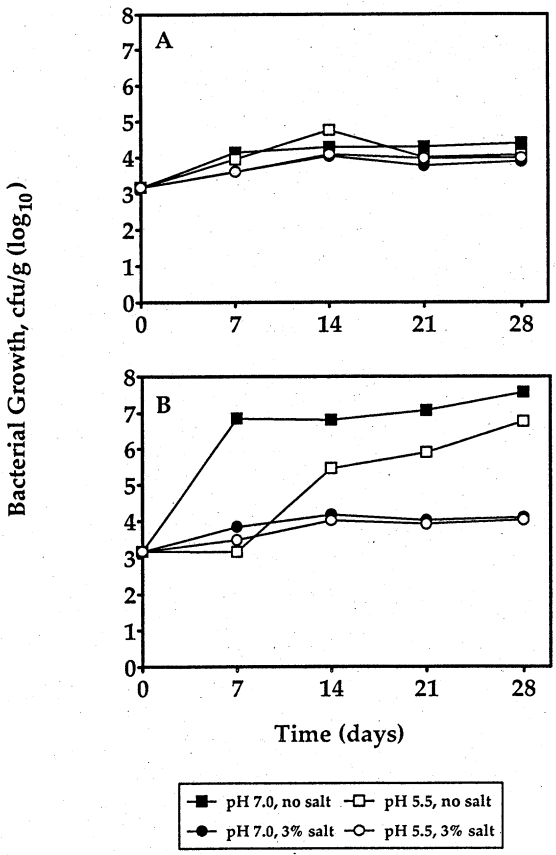

FIG. 3. THE EFFECT OF STATIC TEMPERATURE ABUSE ON GROWTH OF *CLOSTRIDIUM PERFRINGENS* FROM A SPORE INOCULUM IN VACUUM-PACKAGED, COOK-IN-BAG GROUND BEEF, THAT INCLUDED 0.3% SODIUM PYROPHOSPHATE AT pH 5.5 OR 7.0, AND SALT LEVELS 0 OR 3%, STORED AT 4C

The samples were moved to 28C for 15 h (A) or 20 h (B) before plating on their scheduled sampling day (7, 14, 21, and 28 days).

ing day the *C. perfringens* population in all beef samples did not increase, regardless of pH or salt levels (Fig. 3). When samples were transferred 20 h before plating to 28C, the organism grew to > 6 log_{10} cfu/g, in beef samples at pH 7.0 or 5.5 in the absence of salt. There was no increase in the numbers of organisms in samples that contained 3% salt (Fig. 3). To determine the effect of cyclic temperature abuse, samples stored at 4C were transferred to 28C for 12 or 24 h, 7 days before their scheduled sampling day (7, 14, 21, and 28). Samples

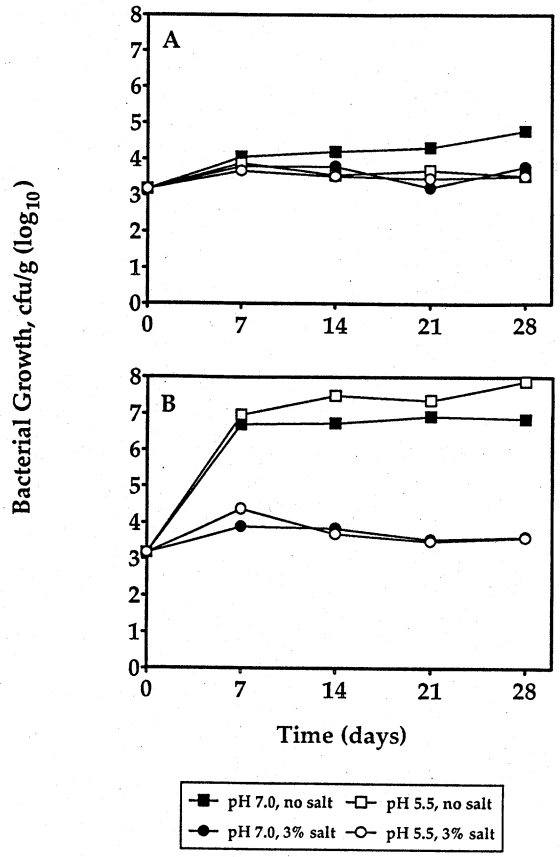

FIG. 4. THE EFFECT OF CYCLIC TEMPERATURE ABUSE ON GROWTH OF *CLOSTRIDIUM PERFRINGENS* FROM A SPORE INOCULUM IN VACUUM-PACKAGED, COOK-IN-BAG GROUND BEEF, THAT INCLUDED 0.3% SODIUM PYROPHOSPHATE AT pH 5.5 OR 7.0, AND SALT LEVELS 0 OR 3%, STORED AT 4C
The samples were moved to 28C for 12 h (A) or 24 h (B), 7 days before plating on their scheduled sampling day (7, 14, 21, and 28 days).

abused for 12 h showed little or no growth regardless of pH or salt level. However, when abused for 24 h at either pH salt-free samples at both pH values resulted in cell populations of > 6 log_{10} cfu/g (Fig. 4); there was no growth when salt was present.

The thermal resistance of *C. perfringens* spores (expressed as D-values in min) in beef slurry that included 0.3% sodium pyrophosphate at pH 5.5 or 7.0, and salt levels 0 or 3% are shown in Table 2. The D-values were significantly decreased

TABLE 2.
MEAN[a] D-VALUES ± STANDARD DEVIATION AT 99C OF SPORE COCKTAIL OF *CLOSTRIDIUM PERFRINGENS* STRAINS NCTC 8238, NCTC 8239 AND ATCC 10288 SUSPENDED IN SLURRY THAT INCLUDED 0.3% SODIUM PYROPHOSPHATE AT pH 5.5 OR 7.0 WITH (3%) OR WITHOUT SALT

Beef Product		
pH	Salt (%)	D-value at 99C (Min)
7	3	$23.3^{b} \pm 1.4$
7	0	$19.8^{b,c} \pm 2.1$
5.5	0	$17.3^{b,c} \pm 0.1$
5.5	3	$14.0^{c} \pm 1.7$

[a]Means represent two replications.
[b-c]Means in column with different superscript are significantly different ($p < 0.05$).

($p < 0.05$) from 23.33 (pH 7.0, 3% salt) to 13.99 min (pH 5.5, 3% salt) at 99C. In a study by Bradshaw *et al.* (1977), D-values at 99C for *C. perfringens* spores suspended in commercial beef gravy ranged from 26 to 31.4 min. The lower D-values obtained in our study may be, primarily, attributed to the heating menstruum. Microorganisms usually have their maximum heat resistance at pH values close to neutrality and the resistance decreases as pH moves to acidity (Montville and Sapers 1981; Rodrigo *et al.* 1990). Presence of salt results in spore dehydration, which accounts for increased heat resistance. Gould and Dring (1975) suggested that developing and maintaining extreme thermal resistance of bacterial endospores depends on the ability to maintain relatively dry protoplasts through an osmotic function of the surrounding expanded cortex.

While a combination of 3% salt and pH 5.5 can significantly decrease the heat resistance of *C. perfringens* spores at 99C, mild heat treatments given to foods that are packaged and then cooked will not eliminate *C. perfringens* spores. This heat treatment may be adequate to destroy the vegetative cells of spoilage and pathogenic foodborne pathogens, but could serve as the spore activation step. In a study by Barnes *et al.* (1963), about 3% of spores germinated in raw beef without prior heat shock, but almost all germinated after the meat was heated. Spores germinate at a reduced rate without prior heat shock (Craven 1980). Desirable organoleptic attributes of foods are unlikely to be retained if the thermal process is designed to inactivate *C. perfringens* spores.

The present study indicated that the organism may grow to unsafe levels if *sous vide* products are poorly handled or temperature abused for a relatively long period.

For vacuum-packaged, cook-in-bag beef products that included 0.3% sodium pyrophosphate, cyclic and static temperature abuse for $\leqslant$ 15 h did not lead to *C. perfringens* growth from spore inocula. An extra degree of safety may be assured in such products by supplementation with salt at 3% level.

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